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14. ABSTRACT
We previously showed that constitutive activation of the PDGF beta receptor (PDGFR) in mortal human fibroblasts (HDFs) by certain viral oncoproteins results in partial transformation of these cells. However, two weeks after reaching their peak density, these partially transformed cells produce a small (<3 kDa) peptide that induces massive apoptosis in an autocrine manner. Specifically, this peptide induces apoptosis by promoting Bax activation and subsequent mitochondrial dysfunction. The primary goal of this project has been to identify the apoptotic peptide produced by these cells. Our recent evidence suggests that production of this peptide requires specific PDGFR signaling pathways and a matrix metalloprotease (MMP). Furthermore, we found that the peptide is negatively charged and can be competitively inhibited by a peptide derived from the N-terminus of MMP-2. Our evidence also implicated the proapoptotic protein p66Shc and increased levels of intracellular ROS in apoptosis of HDFs. We therefore speculate that that the apoptotic peptide is a degradation product of an extracellular protein that binds to a cell surface receptor and enters the cell via endocytosis. We propose that the peptide, once inside the cell, affects the activity of p66Shc, which in turn promotes production of intracellular ROS followed by Bax activation and apoptosis. Although we have not yet identified the apoptotic peptide, we have discerned some of its unique characteristics and effects. We are optimistic that the information gained from these studies will allow us to identify this peptide in the future. Once identified, this peptide could be used to design a novel anti-cancer therapeutic.

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INTRODUCTION:

We have been investigating the effect of sustained activation of the platelet-derived growth factor beta receptor (PDGFR) in mortal human fibroblasts (HDFs). The small bovine papillomavirus E5 oncoprotein and v-Sis (the viral homolog of PDGF-B) is able to crosslink and constitutively activate the PDGFR and thereby induce a subset of transforming phenotypes when expressed in HDFs [1]. However, two weeks after they reach their peak density HDFs partially transformed by E5 or v-Sis undergo massive apoptosis [3]. Our evidence suggests that these cells produce a small peptide that induces apoptosis in an autocrine manner. Specifically, this peptide induces a type of caspase-independent, Bcl-2-resistant apoptosis by promoting mitochondrial dysfunction, which results in the release of the apoptotic mitochondrial protein AIF into the cytosol and its subsequent translocation to the nucleus [3]. We hypothesize that HDFs produce a peptide that indirectly activates the proapoptotic protein Bax, which in turn increases the mitochondrial membrane permeability and subsequent apoptosis. We believe HDFs produce this peptide as a negative feedback response to sustained PDGFR activation to prevent full-scale transformation. The primary goal of this project has been to identify the apoptotic peptide produced by partially transformed HDFs. Since this peptide can induce apoptosis of a number of different tumor cell lines including MCF-7 and MDA human breast carcinoma cells, once identified it could serve as the forerunner of a novel anti-breast cancer agent.

FINAL PROGRESS REPORT:

Extracellular peptides associated with apoptosis of E5-expressing HDFs.

We attempted to directly identify the apoptotic peptide by searching for peptides that are present in apoptotic medium but not control medium. The overall strategy used is illustrated in Figure 1. Briefly, the low molecular weight medium fraction containing the apoptotic activity was isolated using centrifugal filtration devise with a molecular weight cut off size of 3 kilodaltons (kDa) and then concentrated three-fold. The low molecular weight fraction of medium from live control cells was isolated and processed in the same manner. The <3kDa medium fractions were then sent to Proteomic Research Services (Ann Arbor, MI) for MALDI/MS and LC/MS/MS analyses. MALDI/MS profiles and LC/MS/MS results were compared between the apoptotic and control samples.

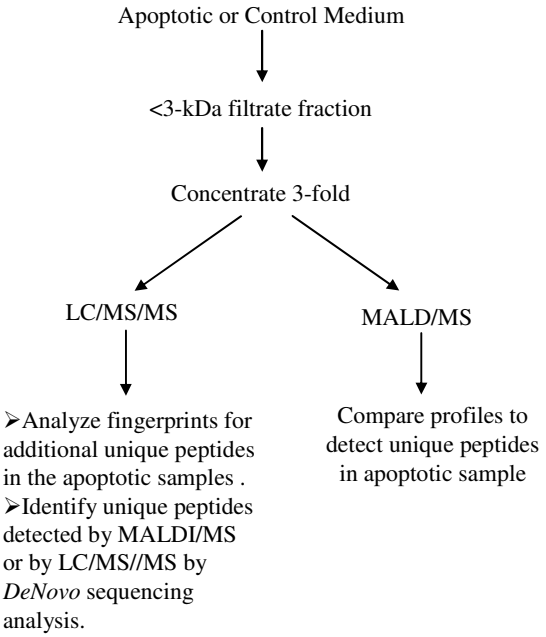


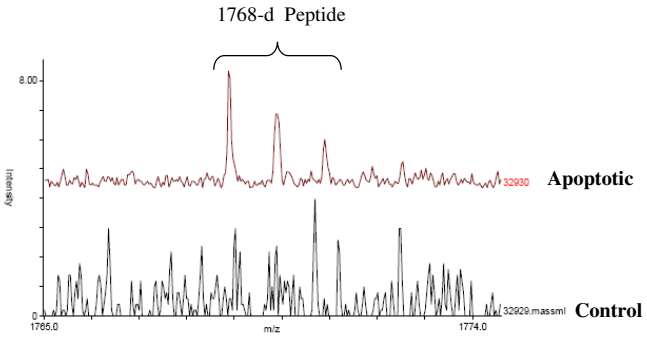
Figure 1. Strategy for identification of peptides present in apoptotic medium from E5-HDFs.

Comparison of the MALDI/MS profiles revealed a peptide with a molecular mass of 1768 daltons and a mass:charge ratio of 885.01 present in the apoptotic medium but not in the control medium (Figure 2A). By subsequently committing LC/MS/MS data to *DeNovo* sequencing analysis the sequence of this peptide was determined to be: N-**(HA)PPPQPPRPQPPPQQ-C,** where the N-terminal amino acids are either HA or AH (Figure 2B). This peptide has significant homology to the N-terminus of fibrillin-2, a component of extracellular microfibrils in connective tissue. The 16-mer peptide having

HA at its N-terminus subsequently was chemically synthesized and tested for apoptotic activity on HDFs. Unfortunately, this peptide did not promote death of HDFs (data not shown), suggesting that it is not the apoptotic peptide produced by partially transformed HDFs. In the future we plan to test the apoptotic activity of the alternative peptide which has AH at its N-terminus.

Additional peptides differentially present in the apoptotic medium were detected by LC/MS/MS analysis and are listed in Table 1. Although LC/MS/MS analysis attempts to match a particular peptide to a known sequence in the data base, the matches for all but two of the unique peptides in the apoptotic medium received a low probability score and therefore were unlikely to be true matches. One of the two remaining peptides, a 1370.67 dalton peptide, matched a sequence within the pleckstrin homology domain of cytohesin-4, a guanine nucleotide exchange factor involved in vesicle formation and trafficking. This match received an intermediate probability score, and so, the likelihood of it being a true match is uncertain. The other peptide had a mass of 1656.98 daltons and matched a sequence contained within the zymogen form of matrix metalloproteinase-2 (proMMP-2 or gelatinase A), a secreted protease which binds to and degrades components of the extracellular matrix, particularly denatured collagen. This match received a relatively high probability score and is likely to be a true identity. The peptide specifically matched a sequence corresponding to

A.



B.

1768-d peptide: **HAPPPQPPRPQPPPQQ**

Fibrillin-2: **QPPPPKPPRPQPPPQQ**

Figure 2. Identification of a small peptide specific to apoptotic medium from E5-HDFs. A. MALDI/MS profiles of low molecular weight fractions from Apoptotic and Control medium showing the presence of a 1768-dalton (d) peptide in the Apoptotic but not the Control medium. **B.** The sequence of the 1768-dalton peptide was determined by committing LC/MS/MS data for the Apoptotic sample to PEAKS software for Auto-*DeNovo* sequencing analysis followed by manual validation. The sequence of this peptide is shown in red in comparison with the highly homologous sequence of the N-terminus of Fibrillin-2.

Table 1. Peptides Specifically Detected in the <3kDa Fraction of Apoptotic Medium

Mr (daltons)	Predicted peptide sequence	Protein match
1768.00	(HA)PPPQPPRPQPPPQQ*	Fibrillin-2, N-terminus
1656.98	IIKFPGDVAPKTDKE**	proMMP-2, N-terminus
1370.67	ENLSVQKVDDPK***	Cytohesin-4 (pleckstrin homology, Sec7 and coiled/coil domains 4)
2042.06	Matches not significant	
1343.64	Matches not significant	
1307.67	Matches not significant	
1122.54	Matches not significant	

* identified by *DeNovo* sequencing

**received a high probability score of 50 and low expect value (the number of times we would expect an equal or higher score purely by chance) of 0.15, which is significant

***received an intermediate probability score of 30 and expect value of 45, which may or may not be significant.

the N-terminus of the propeptide of this enzyme. This particular region of MMP-2 binds intramolecularly to a fibronectin type II (FN2) module adjacent to the catalytic domain and thereby induces a conformational change that inhibits the catalytic activity of the enzyme [2, 4, 5]. Cleavage of the N-terminal pro-peptide region by a cell surface-associated, membrane-type (MT)-MMP and other proteases relieves the allosteric inhibition, thereby activating the enzyme [2, 4]. Since MMP-2 is normally secreted by fibroblasts and its N-terminal propeptide domain is susceptible to multiple cleavage events, it stands to reason that a peptide derived from the N-terminus of MMP-2 would be present in the conditioned medium of HDFs.

Therefore, we decided to determine whether or not the pro-MMP-2 derived peptide played a role in apoptosis of the E5-HDFs. First, a synthetic form of the peptide was added to HDFs at various concentrations. However, the peptide displayed no apoptotic activity, even at a concentration of 400 μg/ml (data not shown). This suggests that this peptide is not the apoptotic peptide produced by partially transformed HDFs. Surprisingly, we found that prolonged treatment of HDFs with the pro-MMP-2-derived peptide inhibited apoptosis (data not shown). Unlike the E5 transformed HDFs, normal HDFs do not undergo apoptosis two weeks after confluence [3]. However, these cells eventually die within 50-60 days if cultured without a medium change. Apparently, the pro-MMP-2 peptide inhibited death of normal HDFs due to long-term culture. It is possible that the normal HDFs, like the E5-transformed HDFs, produce the apoptotic peptide, but do so after a much longer period in culture. If this is the case, the pro-MMP-2 peptide should inhibit the activity of the apoptotic peptide produced by the E5-HDFs. To test this possibility, normal HDFs were treated with the <3kDa apoptotic medium fraction in the absence or presence of increasing concentrations of the pro-MMP-2 peptide. Several days later the viability of the cells was assessed as shown in Figure 3. As expected and shown previously [3], the <3kDa apoptotic fraction efficiently killed the cells, reducing the viability to less than 2%. However, the presence of the pro-MMP-2-derived peptide reduced the ability of the <3kDa medium fraction to kill the cells, enhancing the viability of the cells in a dose-dependent manner. A control peptide (the fibrillin-2-like peptide also found in the conditioned medium) when used at a relatively high concentration only minimally reduced the apoptotic activity of the <3kDa medium fraction. Therefore, the pro-MMP-2 peptide specifically and dose-dependently reduced the death-inducing activity of the apoptotic peptide.

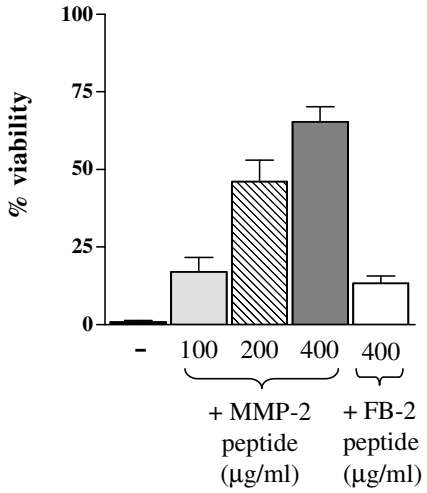


Figure 3. The MMP-2-derived peptide inhibits the apoptotic activity in the conditioned medium of E5-HDFs. The <3kDa fraction from the apoptotic medium was added to normal HDFs in the absence (-) or presence of the MMP-2-derived peptide or the Fibrillin-like (FB-2) peptide at the indicated concentrations. (The sequence of both peptides is shown in Table 1). Several days later the cells were trypsinized and stained with trypan blue and counted for determination of cell viability. Viability was determined as the percent of unstained live cells versus the total number of cells. Mean values with standard error from cells treated in triplicate are shown.

The pro-MMP-2-derived peptide could inhibit the apoptotic peptide by directly interacting with it or by competitive inhibition, i.e., binding to but not affecting the target of the apoptotic peptide. We believe the latter possibility to be the case, since pre-incubating the apoptotic medium fraction with the pro-MMP-2-derived peptide did not enhance the inhibitory effect of the pro-MMP-2 peptide (data not shown). Nonetheless, in either case, we may be able to identify the apoptotic peptide by

identifying proteins that interact with the pro-MMP-2 peptide. Even if the pro-MMP-2 peptide is a competitive inhibitor, identifying proteins that interact with it may allow us to identify the target of the apoptotic peptide. Once identified, the target then could serve as an affinity ligand for subsequent purification of the apoptotic peptide. Moreover, knowledge of this target should provide insight into the apoptotic peptide's mode of action.

If the pro-MMP-2-derived peptide is a competitive inhibitor and binds to the same target as the apoptotic peptide, it stands to reason that the two peptides may be similar with respect to their amino acid sequence. After performing a BLAST search, we found that amino acids 843-852 of collagen type 18 shared 80% sequence identity with the pro-MMP-2 derived peptide (Figure 4). Collagen 18, a proteoglycan collagen, is an extracellular matrix component of basement membranes. The anti-angiogenic peptide endostatin is a C-terminal fragment of collagen 18 generated after cleavage by extracellular proteases such as MMPs and cathepsin-L [6]. Although the region homologous to the pro-MMP-2 peptide is not in the endostatin domain of collagen 18, it would be reasonable to assume that this collagen may be proteolytically cleaved at other sites to generate other bioactive peptides, including an apoptotic peptide. In fact, the region of homology with the pro-MMP-2 peptide is flanked by two motifs resembling MMP cleavage sites (Figure 4)[7]. Consistent with the notion that the

Figure 4. Sequence alignment of the pro-MMP-2-derived peptide and a region in collagen type 18. Identical amino acids are indicated by lines between the sequences. Underlined sequences in collagen 18 resemble MMP cleavage sites and arrows indicate site of cleavage. The sequence in red comprises a peptide that is 1370.68 daltons, which is the same size of one of the peptides detected exclusively in the apoptotic medium. Numbers at the top refer to the position of amino acids in collagen 18.

apoptotic peptide is derived from an MMP cleavage product of collagen 18, we recently found that MMP's are required for generation of the apoptotic peptide, as both pan-MMP and MMP-2-specific inhibitors inhibited production of the peptide by E5-HDFs (Table 2). Moreover, a peptide corresponding to residues 839-852 of collagen type 18, which includes the pro-MMP-2- homologous domain, has a predicted molecular weight of 1370.68 daltons, which is nearly the same mass as one of the peptides differentially produced by the E5-HDFs (Table 1). Therefore, we tested the apoptotic activity of a synthetic peptide corresponding to residues 839-852 of collagen type 18 (Figure 4, sequence in red). Unfortunately this synthetic peptide did not induce apoptosis of HDFs. We also plan to test the apoptotic activity of a longer cleavage product (corresponding to residues 839-867) of collagen 18 which contains the region of homology with the MMP-2-derived peptide.

We also obtained evidence that the apoptotic peptide has an overall negative charge in its active form. Pre-incubation of the <3 kDa apoptotic medium fraction with a positively charged nylon membrane substantially reduced the apoptotic activity of the medium fraction, while pre-incubation with nitrocellulose had no effect on the apoptotic activity (Figure 5). This suggests that the apoptotic peptide has an overall negative charge and was depleted from the medium by binding to the positively charged membrane in an electrostatic manner. This is consistent with our previous observations that the apoptotic peptide is not active at a pH below 7.5 (suggesting that its isoelectric point is lower than 7.5) and cannot be depleted from the medium with negatively charged heparin sulfate agarose beads.

Since the apoptotic peptide is likely to be negatively charged, we attempted to purify it by anion exchange chromatography. Specifically, the <3kDa fractions from control and apoptotic mediums were loaded onto Vivapure Maxi Q M spin columns (Sartorius), which contain a strong basic anion

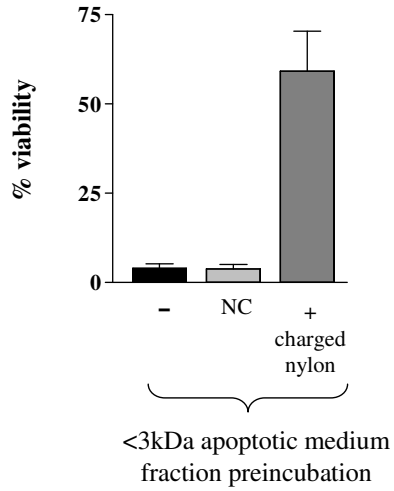


Figure 5. Depletion of the apoptotic activity from the <3kDa conditioned medium fraction with a positively charged nylon membrane. The <3kDa apoptotic medium fraction was either untreated (-) or pre-incubated with nitrocellulose (NC) or a positively charged nylon membrane for several hours. The untreated or treated apoptotic medium was added to HDFs in duplicate by medium replacement. Several days later the cells were assessed for viability by trypan blue dye exclusion assay. Values shown are the mean percent viability with standard error.

exchanger (quaternary ammonium) incorporated into a membrane as its chromatography matrix. After centrifugation at 2000 x *g* and washing, the spin columns were eluted with 0.5-1M NaCl. However, we were unable to recover apoptotic activity from the eluate, suggesting that the elution was not efficient and the apoptotic peptide remained bound to the column. Future experiments are planned to optimize the elution conditions. Once bound peptides are successfully eluted from the membrane, eluates will be subjected to LC/MS/MS analysis. Bound peptides present in the apoptotic medium but not the control medium would be reasonable candidates for the apoptotic peptide.

Intracellular signaling events associated with apoptosis of E5-expressing HDFs.

Elucidation of the signaling events involved in the production of the apoptotic peptide as well as the downstream signaling pathways elicited in response to the peptide should provide important clues regarding this peptide's identity. Our evidence suggested that signaling from the PDGFR is required for apoptosis of E5-expressing HDFs [3]. In an effort to delineate which PDGFR signaling pathways are associated with the apoptotic phenotype, we utilized two partially defective E5 mutants, C39S and W32S. These mutants were originally described as being defective for transformation of mouse C127 fibroblasts but still able to bind to and activate the PDGFR [8, 9]. When expressed in HDFs, we found that the W32S and C39S mutants were capable of interacting with the PDGFR (Figure 6A), but their ability to promote receptor tyrosine phosphorylation was reduced (Figure 6B). Phenotypically, HDFs expressing the C39S or W32S mutant appeared morphologically transformed and were able to proliferate beyond their normal saturation density like wild type E5-expressing HDFs (data not shown). However, the

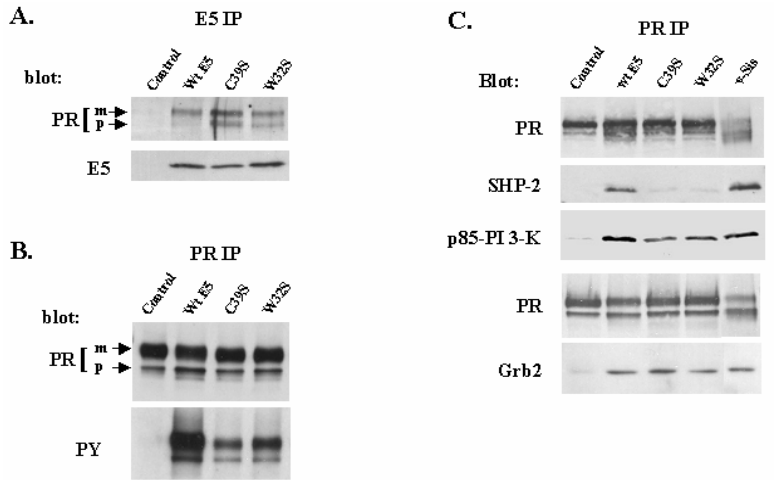


Figure 6. The effect of the C39S and W32S E5 mutants on tyrosine phosphorylation of the PDGFR and its ability to recruit various substrates. PDGF receptor (PR; **B** and **C**) or E5 (**A**) was immunoprecipitated (IP) from extracts of HDFs expressing wild type (Wt) or mutant (C39S, W32S) E5, or no viral oncogene (control). IPs were subjected to PDGF receptor, E5, anti-phosphotyrosine (PY), p85-PI3'-kinase (PI3-K), SHP-2, or Grb-2 immunoblotting (blot) as indicated. Mature (m) and incompletely processed precursor (p) forms of PR are indicated.

mutant E5-expressing HDFs were severely defective for loss of contact inhibition (focus formation) and displayed delayed apoptosis relative to the wild type E5-expressing cells (data not shown). In addition, we found that these mutants did not globally disable the PDGFR from recruiting substrates, as certain substrates were better able to bind to the receptor in the presence of these mutants than others. For example, coimmunoprecipitation analysis revealed that the C39S and W32S mutants were able to promote substantial binding of PI3'-kinase and Grb-2 to the receptor but were severely defective for promoting recruitment of SHP-2 to the receptor (Figure 6C). Thus, recruitment of SHP-2 to the PDGFR correlated with focus formation and apoptosis of the E5-expressing HDFs.

To initially characterize the SHP-2- mediated pathway that may be involved in focus formation and/or apoptosis of HDFs, we attempted to identify its associated proteins in the E5-expressing HDFs, since SHP-2 has been proposed to function as a signaling adaptor [10, 11]. Coimmunoprecipitation analysis revealed that the pro-apoptotic 66-kDa isoform of Shc could form a stable complex with SHP-2 in the E5- and v-Sis-expressing HDFs but not in the control HDFs (Figure 7A). Moreover, very little p66Shc was complexed with SHP-2 in HDFs expressing the C39S and W32S E5 mutants. Therefore, an interaction between SHP-2 and p66Shc occurs in response to sustained PDGFR activation in HDFs and correlates with recruitment of SHP-2 to the activated PDGFR. This suggests that p66Shc is recruited to the receptor via SHP-2. Moreover, given the phenotype of the C39S- and W32S-expressing HDFs, the presence of the SHP-2-p66Shc complex also correlates with focus formation and apoptosis of HDFs.

The p66Shc isoform differs both structurally and functionally from its 52- and 46-kDa splice variants (reviewed in [12]). p66Shc has been shown to play a role in apoptosis by translocating to the mitochondria and promoting the generation of intracellular reactive oxygen species (ROS) [13-15]. p66Shc possesses an additional N-terminal domain, and phosphorylation of Ser^{36} within this domain is required for its oxidant-inducing and proapoptotic activity [16-18]. Therefore, we examined the status of p66Shc-Ser^{36} phosphorylation in HDFs expressing the different E5 proteins. Briefly, Shc was immunoprecipitated from cell extracts and then subjected to immunoblotting using a phospho-Ser^{36}-p66Shc-specific antibody. As shown in Figure 7B, p66Shc-Ser^{36} phosphorylation was increased substantially in the E5- and v-Sis-expressing HDFs compared to control cells, suggesting that p66Shc is phosphorylated at Ser^{36} in response to sustained PDGFR activation in HDFs. However, no increase in Ser^{36} phosphorylation was observed in HDFs expressing the C39S or W32S E5 mutant. Therefore, Ser^{36} phosphorylation of p66Shc in HDFs correlates with recruitment of SHP-2 to the PDGFR, SHP-2-p66Shc complex formation, focus formation and apoptosis.

To determine if SHP-2 is required for Ser^{36} phosphorylation of p66Shc, p66-Ser^{36} phosphorylation

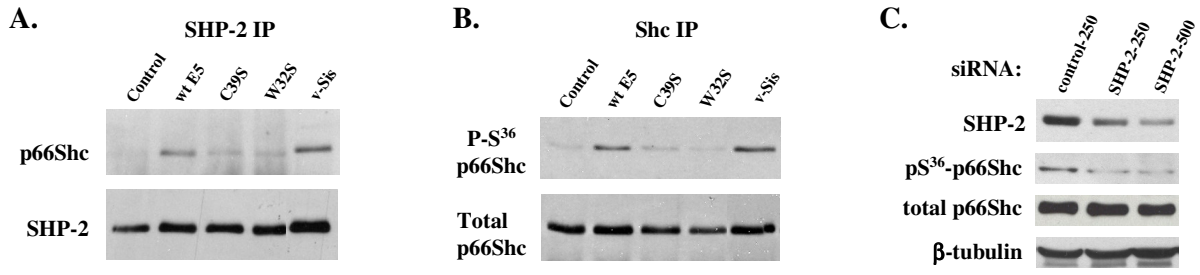


Figure 7. p66Shc associates with SHP-2 and is phosphorylated on Ser^{36} in response to PDGFR activation in HDFs. **A & B.** HDFs expressing the indicated E5 protein, v-Sis or no viral oncoprotein were subjected to SHP-2 (**A**) or Shc (**B**) immunoprecipitation (IP) followed by immunoblotting for Shc, SHP-2 or Ser^{36} -phosphorylated p66Shc as indicated. **C.** E5-expressing HDFs were transfected with 250 or 500 pmoles of a control or SHP-2-specific siRNA and then examined for levels of SHP-2 expression, Ser^{36} phosphorylation of p66Shc, total p66Shc, or β-tubulin by immunoblotting.

was examined after transient siRNA-mediated knockdown of SHP-2 expression in the E5-expressing HDFs. Briefly, E5-expressing HDFs were transiently transfected with a control or SHP-2 siRNA (Santa Cruz Biotechnology, Inc. products sc-37007 and sc-36488, respectively). Forty-eight hours after transfection, cells were lysed and examined for Ser^{36} phosphorylation of p66Shc and SHP-2 expression by immunoblotting. As shown in Figure 7C, the SHP-2 siRNA reduced the levels of SHP-2 expression as well as Ser^{36} phosphorylation of p66Shc in the E5-expressing HDFs. This suggests that SHP-2 plays a role in phosphorylation of p66Shc on Ser^{36} in response to PDGFR activation. Taken together, our results suggest that sustained activation of the PDGFR in HDFs promotes Ser^{36} phosphorylation of p66Shc by recruiting p66Shc to the receptor via an interaction with SHP-2. We speculate that once associated with SHP-2 at the receptor, p66Shc is proximal to a membrane-associated kinase that promotes its phosphorylation. Once phosphorylated on Ser^{36}, p66Shc could play a role in apoptosis of the E5-expressing HDFs.

Since p66Shc is involved in promoting the generation of intracellular ROS, we measured the level of ROS in the E5-expressing HDFs using the oxidant-sensitive fluorescent dye Redox Red (Molecular Probes). Subconfluent or confluent E5-expressing HDFs were treated with Redox Red, trypsinized and analyzed by flow cytometry. Figure 8 shows that the level of intracellular Redox Red-reactive ROS in the E5-expressing HDFs increased when the cells reached confluence. Furthermore, conditioned medium from confluent E5-expressing HDF induced intracellular ROS production in subconfluent E5 HDFs (Figure 8). These results suggest that a factor released by the E5-expressing HDFs prior to or at confluence (possibly the apoptotic peptide) promotes the generation of intracellular ROS in an autocrine manner.

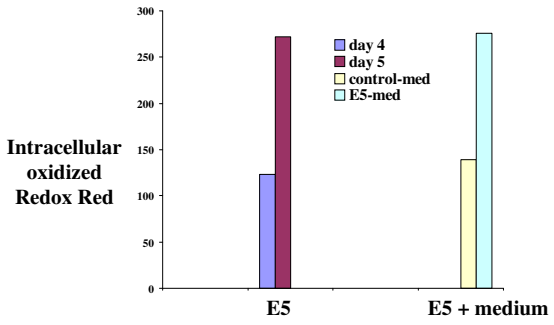


Figure. 8. Density-dependent increase in intracellular ROS levels in the E5-HDFs. Left: E5-expressing HDFs were treated with Redox Red (Molecular Probes) 4 and 5 days after plating. **Right:** Subconfluent E5-expressing HDFs were subjected to medium replacement with medium from either confluent control cells (contro-med) or confluent E5-expressing HDFs (E5-med) and then one day later treated with Redox Red. After Redox-Red treatment cells were trypsinized and analyzed by flow cytometry for quantification of mean intracellular fluorescence.

Next, we examined the activity of signaling intermediates downstream of the PDGFR in the E5- and v-Sis-expressing HDFs. Specifically, we ascertained the phosphorylation status of AKT, JNK, ERK1/2, and Fyn/Src as an indication of their activity by immunoblot analysis using phospho-specific antibodies (Figure 9). Surprisingly, phosphorylation of ERK1/2 (which could occur downstream of Grb2) did not increase in response to E5 or v-Sis expression in HDFs. In contrast, phosphorylation of AKT (which occurs downstream of PI3'-kinase) and JNK (which could occur downstream of Grb2 or PI3'-kinase) was substantially increased in the E5- and v-Sis-expressing HDFs compared to the control cells. In addition, phosphorylation of c-Jun, a major substrate for JNK, was also increased in these cells (data not shown), confirming that the activity of JNK was augmented. We also found that inhibitory phosphorylation of Fyn and/or Src was decreased in response to E5 and v-Sis expression in HDFs, suggesting that Fyn/Src is also activated in these cells. Thus, activation of AKT, JNK, and Fyn/Src but not ERK1/2 occurs in response to sustained PDGFR activation in HDFs. To determine if these kinases are required for apoptosis of E5-expressing HDFs, we tested the effect of specific pharmacological

inhibitors on this process. Briefly, E5-HDFs were treated with the inhibitors listed in Table 2 prior to confluence and then monitored for signs of apoptosis two weeks later. Preliminary tests verified that these inhibitors were effective at inhibiting their targeted kinase at the concentrations used (data not shown). We found that kinase inhibitors specific for the PDGF receptor, PI3'-kinase, Src, and JNK completely prevented apoptosis (Table 2). Since inhibition of apoptosis occurred only if the inhibitor was added prior to but not after the cells reached confluence, we can conclude that the kinases targeted by these inhibitors are required for production of the apoptotic peptide and not the apoptotic process itself. Taken together, these results suggest that E5-induced activation of the PDGFR and downstream activation of PI3'-kinase-, JNK- and Fyn/Src mediated pathways is required for production of the apoptotic peptide. Consistent with our observation that ERK1/2 was not activated in response to sustained PDGFR activation in HDFs, a MEK1/2-specific inhibitor could not prevent apoptosis of the E5-expressing HDFs (Table 2).

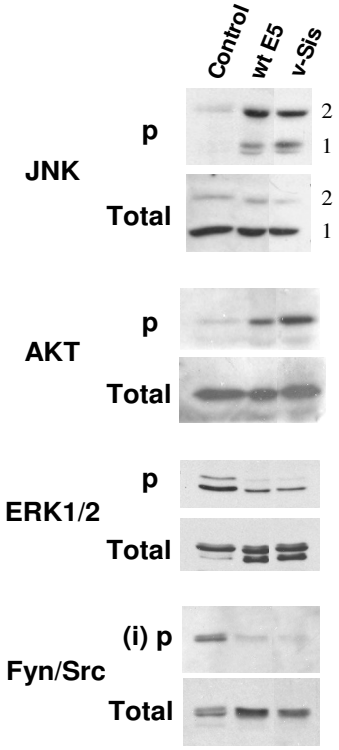


Figure 9. Activation of downstream signaling intermediates in response to sustained PDGFR activation in HDFs. Extracts of HDFs expressing E5, v-Sis, or no viral oncoprotein (Control) were Western blotted for the active, phosphorylated (p) form of JNK, AKT, or ERK1/2 or the inactive (i) phosphorylated form of Fyn or Src. Blots were then stripped and reprobed with pan-specific antibodies detecting the total amount of each protein. JNK isoforms 1 and 2 are indicated on the right.

Table 2. Effect of Various Inhibitors on Apoptosis of E5 HDFs

Inhibitor	Target enzyme	Inhibition* of apoptosis of E5-HDFs
LY294002	PI 3'-kinase	+
Wortmannin	PI 3'-kinase	+
SP600125	JNK	+
Y27362	Rho kinase	delayed
PPI	Src	+
AG1296	PDGF receptor	+
PD98059	MEK-1/2	-
U73122	PLC–γ	-
APHS	Cox2	-
Cyclosporin A	Cyclophillin A	-
MMP inhibitor III	multiple MMPs	delayed
MMP-2 inhibitor	MMP-2	delayed
Furin inhibitor	Furin	-
15dPGJ2	PPARγ; others	+
CAY10410	PPARγ	-

"+" or "delayed" indicates that an inhibitory effect was observed only when the inhibitor was added prior to but not after confluence.

We also found that a general MMP inhibitor as well as a MMP-2-specific inhibitor delayed apoptosis, but only if added prior to confluence (Table 2). This suggests that an MMP, possibly MMP-2, is involved in production of the peptide. On the other hand, inhibition of Furin, another protease involved in post-translational processing of membrane and secreted proteins, did not inhibit apoptosis of E5-expressing HDFs, suggesting that Furin-mediated proteolysis does not play a role in generation of the

apoptotic peptide. Therefore, we speculate that activation of certain PDGFR signaling pathways results in increased expression and/or activation of an MMP, such as MMP-2, which in turn promotes degradation of an extracellular protein leading to production of the apoptotic peptide. It is possible that the apoptotic peptide is a proteolytic product derived from the same region of the MMP-2 prodomain as the inhibitory pro-MMP-2-derived peptide discussed above (Figure 10). Since activation of MMP-2 involves cleavage of its prodomain at several sites [2], multiple peptide cleavage products should be generated. Thus, the apoptotic peptide could be a longer cleavage product containing a domain that binds to a cell surface receptor as well as an apoptotic domain. The inhibitory peptide could be a shorter cleavage product, which contains only the receptor binding domain, and thus could act as a competitive inhibitor. Alternatively, the apoptotic peptide could be a bioactive degradation product of an extracellular matrix protein such as collagen.

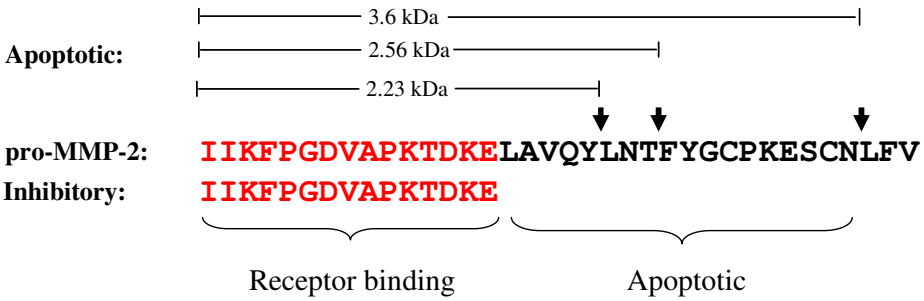


Figure 10. The apoptotic peptide as well as a competitive inhibitor could be derived from the same region within the prodomain of MMP-2. Sequence of amino acids 34-67 of pro-MMP-2 is shown. The peptide which inhibits our apoptotic peptide and longer proteolytic cleavage products that could represent the apoptotic peptide are shown. The apoptotic peptide could comprise a domain that binds to a cell surface receptor and an apoptotic domain. The inhibitory peptide could comprise only the receptor binding domain and thus act as a competitive inhibitor of the apoptotic peptide. Arrows point to cleavage sites for pro-MMP-2 activation [2], and the predicted molecular weights of the resulting cleavage products are indicated above.

We also found that 15d-PGJ_2, a naturally occurring metabolite of prostaglandin J_2, inhibits production of the apoptotic peptide at a concentration of 12.5 μM. E5-transformed HDFs treated with 15d-PGJ_2 prior to but not after confluence were protected from apoptosis. This suggests that 15d-PGJ_2 inhibits the production but not the activity of the apoptotic peptide. 15d-PGJ_2 is a potent agonist for the nuclear receptor PPARγ [19] but also can act in a PPARγ-independent manner by forming adducts with free sulfhydryl moieties of cysteine residues on cellular proteins [20]. Therefore, we tested whether or not CAY10410, a 15d-PGJ_2 analog that is a PPARγ agonist but cannot form adducts with proteins, can inhibit apoptosis of the E5-transformed HDFs. We found that CAY10410 could not prevent apoptosis of these cells, suggesting that 15d-PGJ_2 prevents production of the apoptotic peptide in a PPARγ-independent manner. It is likely that 15d-PGJ_2 forms an adduct with a protein that plays a role in the proteolytic events required for production of the peptide. For example, 15d-PGJ_2 could interact with the sulfhydryl of the critical cysteine residue in the catalytic cleft of an MMP and thereby inhibit its activity.

Summary of key Accomplishments:

- MALDI/MS and LC/MS/MS analyses were able to detect peptides differentially present in apoptotic medium versus control medium. Two of these peptides corresponded to the N-terminus of secreted proteins. One appeared to be derived from the extracellular matrix protein, fibrillin-2, while the other was derived from the pro-form of MMP-2. Other peptides were detected but could not be positively identified.
- Although neither the fibrillin-2-like peptide nor the pro-MMP-2 peptide proved to be apoptotic, the pro-MMP-2 peptide inhibited apoptosis induced by our apoptotic medium fraction. Further evidence suggested that the pro-MMP-2 peptide is a competitive inhibitor of the apoptotic peptide. The pro-MMP-2 peptide could be used to identify the apoptotic peptide's target, which then could be used to purify the apoptotic peptide for subsequent identification. It is possible that the apoptotic peptide is derived from the same prodomain of MMP-2 as the inhibitory peptide. This is consistent with our finding that MMP-2 is required for generation of the apoptotic peptide. On the other hand, the apoptotic peptide could be a proteolytic degradation product of an extracellular matrix protein such as collagen.
- The apoptotic peptide appeared to bind to a positively charged nylon membrane, suggesting that it has an overall negative charge in its active form. The peptide's negative charge will be exploited to more adequately purify the peptide for subsequent identification.
- A metabolite of prostaglandin J2, 15d-PGJ_2, inhibited production of the peptide perhaps by forming an adduct with and inhibiting an extracellular protease that is required for production of the peptide.
- PI3'-kinase-AKT, JNK, and Fyn/Src signaling pathways most likely play a role in production of the apoptotic peptide. We speculate that these pathways are involved in the expression or activation an extracellular protease such as MMP-2, which in turn is responsible for the proteolysis involved in generation of the peptide.
- Ser^{36} phosphorylation of the proapoptotic protein p66Shc and its interaction with SHP-2 occurred in response to sustained activation of the PDGFR in HDFs and correlated with apoptosis of these cells.
- A factor present in the conditioned medium of E5-expressing HDFs enhanced intracellular ROS levels. Thus, it is possible that the apoptotic peptide promotes the generation of intracellular ROS. Since the Ser^{36} phosphorylated form of p66Shc correlated with apoptosis of our cells and has been shown to play a role in apoptosis by inducing mitochondrial hydrogen peroxide production, we speculate that the apoptotic peptide relays a signal to activate p66Shc for generating intracellular ROS.

CONCLUSIONS/MODEL:

The data obtained during this project has allowed us to propose a model (Figure 11) regarding the origin and mode of action of an apoptotic peptide produced by human fibroblasts (HDFs). First, our data suggest that sustained activation of the PDGFR in HDFs leads to activation of PI3'-kinase-AKT, JNK and Fyn/Src pathways, which in turn augment the expression or activity of an MMP, possibly MMP-2. After secretion of this MMP, the apoptotic peptide is generated by MMP-mediated degradation of an extracellular or transmembrane protein. Since the apoptotic peptide is negatively charged, it is unlikely that it enters the cell by passive diffusion through the membrane. Instead, it is more likely that the apoptotic peptide binds to a receptor on the cell surface and enters the cell via receptor-medi-

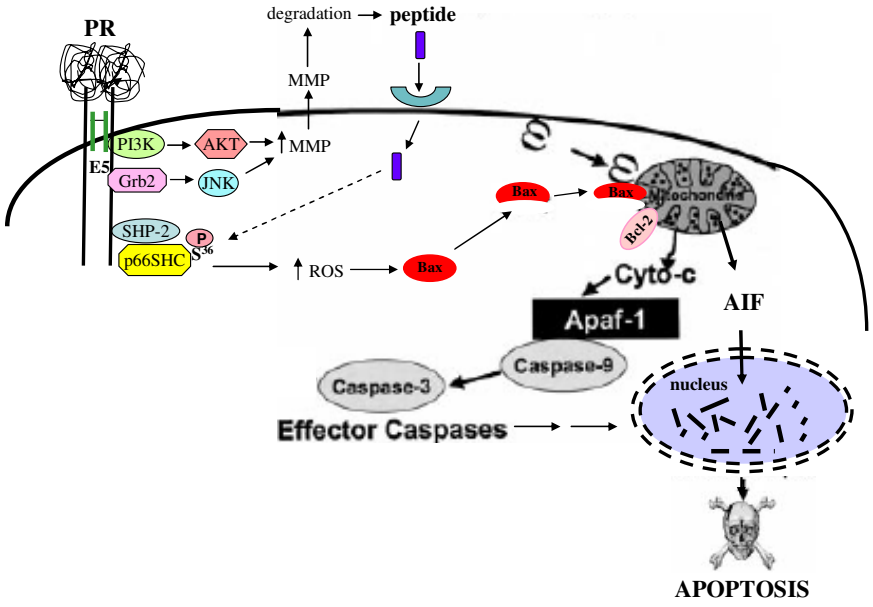


Figure 11. Model of how sustained PDGFR activation in HDFs leads to apoptosis. See text for explanation.

ated endocytosis. Once in the cytosol, the apoptotic peptide then may initiate a signal for p66Shc to promote the generation of intracellular ROS. For example, the peptide could activate the kinase responsible for Ser^{36} phosphorylation of p66Shc, or it could promote p66Shc translocation to the mitochondria, which would result in the production of hydrogen peroxide. An increase in intracellular ROS then could promote activation of a phosphatase such as PP2A, which in turn could dephosphorylate and activate Bax [21]. Once activated, Bax could translocate to the mitochondria and perturb the mitochondrial membrane permeability, allowing the release of cytochrome c and AIF into the cytosol. Activation of the caspase cascade by cytochrome c and translocation of AIF to the nucleus would result in apoptosis. Although we have not yet identified the apoptotic peptide, we have discerned some of its unique characteristics. We are optimistic that the information gained from these studies will allow us to identify this peptide in the future.

Reportable Outcomes (Publication Related to this Project):

Petti, L.M., Ricciardi, E.C., Page, H.J., and Porter, K.A. Transforming signals resulting from sustained activation of the PDGF β Receptor in mortal human fibroblasts. J. Cell. Sci. *In Press*, 2008.

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Lisa M. Petti (PI)

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